

## Emergence of Hepatitis B Virus S Gene Mutant in a Liver Transplant Recipient

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Immunological and genomic analysis of the "a" determinant was carried out in seven patients with concurrent HBsAg and anti-HBs, four of whom were immunized against hepatitis B virus at liver transplant, two with histologically characterized chronic hepatitis B virus infection, and one HBsAg healthy carrier. The immune reactivity of the HBsAg "a" determinant was evaluated by binding to specific monoclonal antibodies, and the corresponding genomic sequence was studied by differential hybridization in microtiter plates and nucleotide sequence analysis. A double mutation generating an amino acid change (glycine to lysine) at residue 145, able to impair recognition by monoclonal antibodies, was observed in the post-transplant serum from one patient. No significant alteration of the "a" determinant sequence or reactivity was detected in the other patients.

Amino acid residue 145 appears therefore to be critical for the recognition by anti-HBs antibodies. A previously undescribed glycine to lysine substitution at this level interferes with the immune reactivity of the "a" determinant.

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**KEY WORDS:** HBsAg "a" determinant, monoclonal antibodies, polymerase chain reaction, DNA enzyme immunoassay, escape mutants

### INTRODUCTION

Antibody (anti-HBs) directed against the HBsAg "a" determinant that is located at residues 124-147 of the HBV S protein is able to confer protective immunity against HBV infection. This domain is relatively conserved and only minor variation is observed among different HBV subtypes. A specific mutation generating an amino acid substitution (glycine to arginine) at position 145 was observed in children vaccinated successfully against HBV. This mutation was able to impair the recognition of HBsAg by a monoclonal antibody

specific for the "a" determinant [Carman et al., 1990]. Although other amino acid changes likely to modify the antigenicity of the "a" determinant have been reported subsequently [Okamoto et al., 1992; Karthigesu et al., 1994; Zuckerman et al., 1994], the mutation at residue 145 appears to be the most frequent worldwide. Indeed, the glycine 145 to arginine substitution was also detected in vaccinated infants from Singapore and from Japan [Harrison et al., 1991; Fuji et al., 1992; Okamoto et al., 1992] and in liver transplant recipients treated with monoclonal antibodies [McMahon et al., 1992].

Since seroconversion to anti-HBs is generally associated with protection toward HBV infection, the concurrence of HBsAg and anti-HBs might indicate the emergence of escape mutants resistant to the host immune response. In this study an immunological and genomic characterization of the "a" determinant were carried out in patients with concomitant HBsAg and anti-HBsAg.

### MATERIALS AND METHODS

#### Patients

Seven patients with concurrent HBsAg and anti-HBs were investigated (Table I). Four patients underwent liver transplantation and showed recurrence of serum HBsAg after initial clearance, and three patients (2 with histologically proven chronic hepatitis and 1 HBsAg healthy carrier) had persistent serum HBsAg together with anti-HBs.

Patients SA, RE, Mo, and Fu underwent liver transplantation for HBV-linked cirrhosis. They were treated with anti-HBs Ig [intravenously at intervention (Hepatect, Biotech, 1000 I.U.) and intramuscularly after surgery (Hepuman B, Berna, 800 I.U.)] and with 40 µg recombinant HBV vaccine (Recombivax HB, Merck Sharp & Dohme). All patients, after initial clearance, had recurrence of serum HBsAg despite the presence of anti-HBs resulting from active and passive immuniza-

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TABLE I. Clinical and Serological Profile of Patients\*

Patient	Sample	ALT	Histology	HBsAg	Anti-HBs (mU/ml)	HBeAg	Anti-HBe	Anti-HBc (IgM)
SA	11.9.91*	53	Cirrhosis	+	-	+	-	+ (-)
	12.9.91	465		-	+(2,620)	N.D.	N.D.	+
	9.4.92	10		-	+(599)	N.D.	N.D.	N.D.
	29.9.92	55		+	+(442)	+	N.D.	+
RE	14.3.92*	46	Cirrhosis	+	-	-	+	+ (-)
	18.3.92	260		-	+(786)	N.D.	N.D.	N.D.
	28.4.92	10		-	+(1001)	-	-	N.D.
	30.9.92	984		+	+(429)	+	-	+
Mo	20.3.91*	N.D.	Cirrhosis	+	-	+	-	+ (-)
	25.3.91	250		-	+	+	+	+
	10.6.91	389		+	+	-	+	+ (-)
Fu	4.3.90*	44	Cirrhosis	+	+	-	+	+
	9.5.90	30		-	+	-	+	- (-)
	3.10.90	45		+	+	-	+	+
	15.5.91	105		+	+	-	+	+
Al	6.2.92	68	CAH	+	+	-	+	+ (-)
	4.6.92	101		+	+	-	+	+ (+)
A22	15.3.89	153	CAH	-	+	-	+	+ (-)
M278	12.1.88	22	N.D.	-	+	+	-	+ (-)

\*Samples analyzed for the immunological and genomic characterization of the "a" determinant are underlined.

\*Samples obtained before transplantation.

\*- titer not available.

\*N.D. = not determined.

tion. Two serum samples were analyzed from each transplanted patient.

Two patients (Al and A22) had histological evidence of chronic active hepatitis, whereas patient M278 was a chronic healthy HBsAg carrier. They showed persistence of serum HBsAg together with anti-HBs. Two distinct serum samples were examined from patient Al.

#### Serological Tests

Tests for HBsAg, anti-HBs, total and IgM anti-HBc, HBeAg/anti-HBe were carried out in duplicate using commercial kits (Abbott Laboratories, North Chicago, IL).

#### Immunological Characterization of HBsAg

The HBsAg "a" determinant was characterized for its differential reactivity in a monoclonal antibody (mAb) binding assay with two specific mAbs, H5 and H35 (kindly supplied by Abbott Laboratories). The "a" determinant mutant containing a glycine to arginine substitution at residue 145 had low affinity for mAb H5, compared to mAb H35. By contrast, wild-type HBsAg strongly bound both mAbs (L. Mimms, pers. comm.).

Serum (200  $\mu$ l) were added to beads coated with mouse monoclonal anti-HBs antibody (Auszyme monoclonal, Abbott Laboratories) and incubated for 16-18 hr at room temperature. After washing, 200  $\mu$ l of mAb (H5 or H35) tagged with biotin were added and incubated for 2 hr at 40°C. After removal of the unbound material and washing, 200  $\mu$ l of rabbit anti-biotin, conjugated with HRPO (AUSAB-EIA, Abbott Laboratories), were incubated 2 hr at 40°C. After washing, the reaction was revealed with OPD and read with a spectrophotometer at 490 nm.

The method for the immunological characterization of HBsAg was evaluated with serum samples characterized previously as wild-type or as containing the amino acid 145 glycine to arginine substitution [Carman et al., 1990].

#### Polymerase Chain Reaction (PCR)

Serum samples (100  $\mu$ l) were heated at 100°C for 10 min and centrifuged for 15 min at 10,000x g. HBV DNA was amplified directly from 5  $\mu$ l of supernatant with PCR primers located at both extremities of the pre-S/S region: A3 (antisense, nucleotide positions 842 to 821, 5'-TTAGGGTTTAAATGTATACCCA-3') and A5 (sense, nucleotide positions 2820 to 2840, 5'-GGGTCACCATATTCTTGGGAA-3'). Restriction sites (HindIII and KpnI) were inserted at the 5' extremities of A3 and A5 primers, respectively, for cloning procedures. PCR was performed in a 100  $\mu$ l volume containing 67 mM Tris-Cl pH 8.8, 16.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.5 mM MgCl<sub>2</sub>, 10 mM 2-Mercaptoethanol, 100  $\mu$ g/ml bovine serum albumin, 0.005% Tween-20, 0.005% Nonidet-P40 (NP40), 200  $\mu$ M of each dNTP, 50 pmoles of each primer, and 2.5 units of thermus aquaticus DNA polymerase (AmpliTaQ DNA polymerase, Perkin-Elmer-Cetus, Norwalk, CT). A DNA thermal cycler (Perkin-Elmer-Cetus) was used to perform 35 cycles of PCR (denaturation: 93°C for 1 min, annealing of primers: 48°C for 1 min, extension: 72°C for 2 min).

#### DNA Enzyme Immune assay (DEIA)

Two ligonucleotide probes, specific for wild-type and mutated sequence of the HBsAg "a" determinant (sub-type ayw), were used for the differential hybridization of PCR products: probe W3 (sense, nucleotide positions 579 to 595, 5'-CTTCGGACGGAAATTGC-3') and probe

TABLE II Reactivity of Serum Samples With mAbs H35 and H5

Sample	Dilution	A <sub>450</sub>		
		H35	H5	H35/H5
wt <sup>a</sup>	1:10	>2	>2	1
	1:100	1.840	0.495	3.7
	1:1000	0.260	0.074	3.5
mut <sup>b</sup>	1:10	>2	0.654	3
	1:100	>2	0.150	13
	1:1000	>2	0.056	35.7
SA 11.9.91	1:100	1.225	>2	0.613
SA 29.9.92	1:100	>2	0.020	100
RE 14.3.92	1:100	>2	>2	1
RE 30.9.92	1:100	>2	0.949	2.1
Mo 25.3.91	1:100	0.030	0.023	1.3
Mo 10.6.91	1:100	>2	>2	1
Fu 4.3.90	1:100	1.366	1.085	1.26
Fu 15.5.91	1:100	>2	>2	1
Al 6.2.92	1:100	>2	>2	1
Al 4.6.92	1:100	>2	>2	1
A22	1:100	>2	>2	1
M276	1:100	>2	>2	1

<sup>a</sup>Wild type, mean of two wt controls.<sup>b</sup>Mutant, mean of three determinations.

M3 (identical to the sequence of probe W3, except for a G to A mutation at nucleotide position 587). Hybridization of PCR products was carried out using the DELTA, a microtiter plate hybridization immunoassay, as described previously [Mantero et al., 1991; Puoti et al., 1992]. The absorbance was read with a spectrophotometer at 450 nm.

### Cloning and Nucleotide Sequence Analysis

The amplified fragments were separated on agarose gel and cloned into the HindIII/KpnI sites of the Bluescript plasmid vector (Stratagene, La Jolla, CA). Nucleotide sequence analysis was undertaken by the dideoxy-chain termination method [Sanger et al., 1977] with the Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, OH). Both strands of each plasmid were sequenced and at least five clones were analyzed from each PCR experiment.

### RESULTS

Serum samples were characterized with mAbs able to discriminate between wild-type and mutant HBsAg. The validation of this method was carried out using serial dilutions of pedigree sera containing wild-type (WT) or mutant viruses [Carman et al., 1990] (Table II). When the absorbance value (A) obtained with mAb H35 was at least six-fold higher than that obtained with mAb H5 on serum diluted 1:100, the sample was arbitrarily considered as potentially positive for the HBV mutant.

All the samples obtained from patients with chronic hepatitis and those derived before liver transplantation reacted strongly with both mAbs, indicating the presence of wild-type "a" determinant. A marked decrease in the affinity for mAb H5, suggesting the presence of mutations in the "a" determinant sequence, was detected in the sample obtained after liver transplantation from patient SA (29.9.92). A less significant de-

crease was observed in post-transplant serum sample from patient RE (30.9.92) (Table II).

The genomic analysis of the HBsAg "a" determinant was carried out by differential hybridization with probes specific for the WT (W3) and for the nucleotide 587-mutant (M3) sequence. The evaluation of the hybridization method was carried out by using serial dilutions of recombinant plasmids containing WT or mutated HBV pre-S/S fragments. By this approach, the cutoff of the method (mean + 3 SD of 20 replicates of negative controls) was established as 0.2 A<sub>450</sub>, and the lower detection limit as 10<sup>3</sup> copies of plasmid present in the initial sample prior to amplification (data not shown).

HBV S gene sequences could be amplified from all serum samples tested. The hybridization of PCR products revealed the presence of wild-type "a" determinant sequences in all samples, with the exception of the post-transplant serum sample of patient SA. This fragment did not hybridize either with the W3 or with the M3 probe. The A<sub>450</sub> values obtained by PCR-differential hybridization of reference plasmids and patients' samples are listed in Figure 1.

The results obtained by differential hybridization were analyzed further by cloning in plasmid vectors and sequencing of amplified DNA fragments. The samples obtained from patients SA and RE were examined before and after transplantation, and as a control, the sample derived from patient A22, strongly hybridizing with the W3 probe. The resulting sequences were compared with the WT consensus sequence and with the mutant derived from the originally described vaccinated infant [Carman et al., 1990].

The pre-transplant serum of patient SA, hybridizing weakly with W3 probe only, contained a mixture of different "a" determinant sequences (Fig. 2). The region corresponding to the W3 probe was identical to the

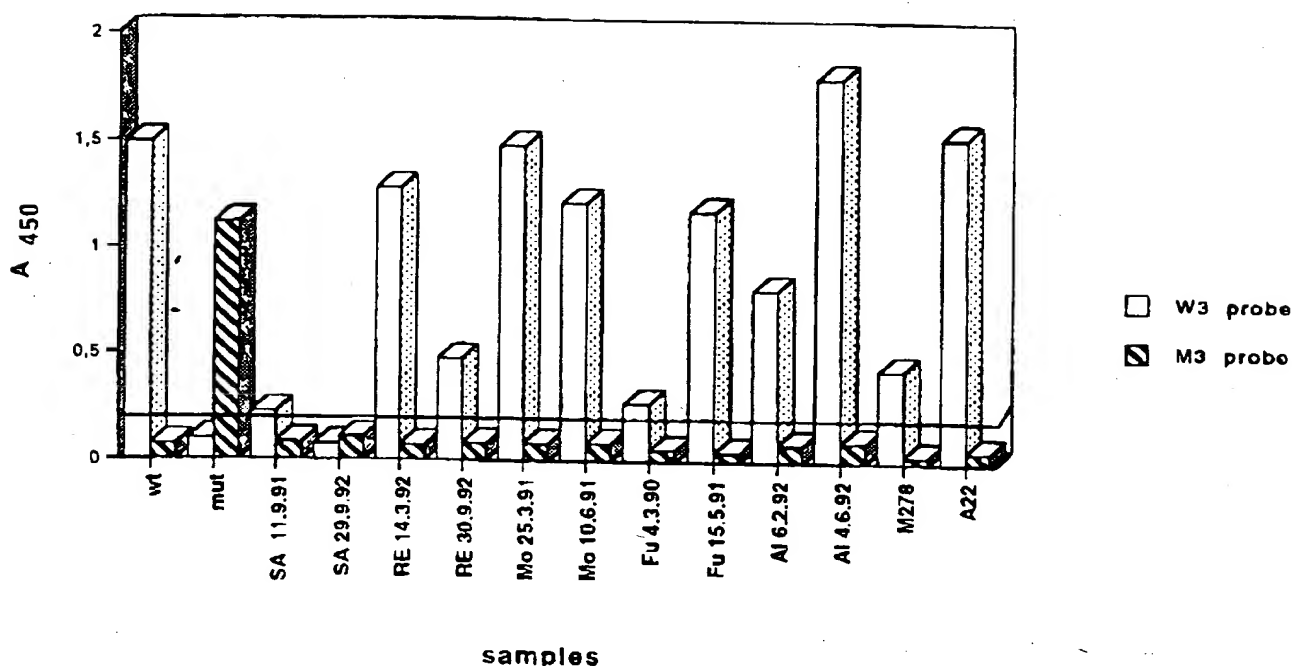


Fig. 1. Absorbance (A450) values obtained after DEIA hybridization with probes W3 and M3 of control plasmids wt and mut ( $10^6$  copies, mean of 10 replicates) and of PCR products obtained from sera of HBV-infected patients. The horizontal line represents the cutoff value of the method.

wild-type sequence in 3 clones, but contained 1 mutation (C to A at position 586, generating an aspartic to glutamic acid change at residue 144) in 3 clones and 2 mutations (the former plus a T to C silent mutation at position 580) in 1. Additional amino acid changes were observed at residues 121 (1 clone), 123 (1 clone), 127 (all clones), 130 (4 clones), 131 (1 clone), 134 (3 clones) and 135 (4 clones).

The posttransplant sample from patient SA, by contrast, contained a homogeneous viral population. The nine clones sequenced were identical to one another, showing the persistence of the threonine at residue 125 and the selection of the tyrosine to serine change at position 134, which was present in three pretransplant clones. The emergence of a double G to A mutation at positions 587 and 588 led to a glycine to lysine change at residue 145 (Fig. 2). This double mutation likely accounted for the lack of hybridization with both W3 and M3 probes (Fig. 1).

Pretransplant serum samples from patient RE contained a homogeneous "a" determinant sequence, with methionine at residue 125 and threonine at residue 127 (both already reported in HBV ayw). Two point mutations, detected in all clones, generated a threonine to isoleucine change at residue 140 not described in other HBV subtypes. Only one clone contained an additional A-C silent mutation at position 589. The 10 clones derived from the posttransplant serum were identical to each other and had the same sequence as the majority of clones obtained before liver transplantation.

The serum of patient A22 contained a mixture of different "a" determinant sequences. All the amino acid substitutions, with the exception of a methionine to isoleucine change at residue 133, were already reported in other HBV subtypes. The sequence corresponding to W3 probe did not show nucleotide substitutions in the majority of clones sequenced (Fig. 2).

## DISCUSSION

Both antibodies [Air et al., 1990] and cytotoxic T-cell response [Pircher et al., 1990] may select viral variants (escape mutants) that are not longer eliminated efficiently by the host immune reaction. Escape mutations selected by antibody response are often located in correspondence of neutralizing epitopes of surface proteins [Kilbourne et al., 1990].

Naturally occurring amino acid substitutions in the immunodominant "a" determinant of HBsAg were shown to interfere with recognition by anti-HBs antibodies [Carman et al., 1990; McMahon et al., 1992]. A G-A mutation at nucleotide position 587 of the HBV S gene, generating a glycine to arginine substitution in the second loop of the "a" determinant (amino acid residue 145 of the S protein), appears to be the most frequent naturally occurring escape mutation in subjects receiving passive and active immunization [Carman et al., 1990; Harrison et al., 1991; Okamoto et al., 1991; Fuji et al., 1992; McMahon et al., 1992].

In the present study, the possible emergence of mutations at codon 145 was examined in patients undergo-

Fig. 2. Nucleotide and amino acid (underlined) sequence of the HHAg "a" determinant (amino acid residues 119-152). The # of clones with each sequence is indicated in brackets. WT: wild-type *avx* consensus sequence (double underline; sequence of probe W3). MUT: nucleotide 587-mutant sequence (double underline; sequence of probe

M3). SA/pre, SA/post: sequence of clones derived from pre- and post-transplant serum samples of patient SA. RE/pre, RE/post: sequence of clones derived from the pre- and post-transplant serum samples of patient RE. A22: sequence of clones derived from the serum sample of patient A22.

ing liver transplantation and in subjects with chronic HBV infection showing concurrent HBsAg and anti-HBs. The antigenicity of the "a" determinant was studied with monoclonal antibodies having differential reactivity toward WT and amino acid 145-mutants. The same serum samples were analyzed for the presence of the mutation at position 587 by means of differential hybridization.

Wild-type antigenicity and pattern of hybridization were detected in all specimens with the exception of a posttransplant serum sample that displayed immunoreactivity and hybridization profiles compatible with the presence of mutations in the "a" determinant. However, the lack of hybridization with the probe specific for the G-A mutation at position 587 excluded the occurrence of this change. It is noteworthy that the pretransplant sample from the same patient showed WT antigenicity despite weak hybridization with the WT

probe. Nucleotide sequence analysis allowed the detection of a C→A mutation at position 586, generating aspartic to glutamic acid substitution at residue 144 of 4/7 of clones derived from the pretransplant serum, whereas all clones obtained from the posttransplant sample contained a double G→A mutation at positions 587 and 588, leading to a glycine to lysine substitution at residue 145. These results confirm the importance of residue 145 for the antigenicity of the "a" determinant and suggest that nonconservative amino acid changes at this level, different from the already described change from aspartic to arginine substitution [Carman et al., 1990; Carman et al., 1991; Fuji et al., 1992; McMahon et al., 1992; Okamoto et al., 1992], are able to alter the immunoreactivity of the molecule. By contrast, the conservative change observed at residue 144 in the pretransplant sample does not appear to modify the antigenicity of HBsAg.

Consistent with hybridization results, nucleotide sequence analysis did not allow the detection of mutations located in correspondence of the probe sequence in the majority of clones derived from the other samples analyzed. In addition, no other substitution of residues critical for the maintenance of "a" determinant conformation [Cashton-Rickardt and Murray, 1989] was observed.

Including the results of this study, the occurrence of the escape mutations of HBsAg has been reported to date only in subjects receiving active or passive immunization. Antibody-driven selection of escape mutants has been demonstrated on infected cell cultures [Air et al., 1990]. The mechanism of variant selection after the polyclonal immune response elicited by vaccination is more difficult to elucidate and can possibly explain the rarity of such variants in vivo. The immunological status of the host, or the immunosuppressive treatment administered after liver transplantation, may play a role in the emergence of escape mutants. Moreover, our results further support the hypothesis that the concurrence of HBsAg and anti-HBs in patients with chronic HBV infection is not related to the selection of variants of the "a" determinant. Other mechanisms, including ineffective immune response directed mainly against heterologous subtype determinants [Shiels et al., 1987; Fiordalisi et al., 1994] or fragments of the pre-S region not involved in viral clearance [Fiordalisi et al., 1994] may play a role in generating this serological profile. However, a larger series of patients should be examined before drawing definite conclusions.

Other naturally occurring HBV S gene variants recently have been reported [Okamoto et al., 1992; McMahon et al., 1992; Karthigesu et al., 1994; Zuckerman et al., 1994], but the epidemiology and possible relevance of these escape mutants in impairing vaccine strategies have still to be determined. Methods for immunological and genomic characterization of the "a" determinant such as those described here may be developed for the detection of novel variants and help understanding the real impact of mutant selection on the natural course of HBV infection.

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